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*J Immunol* 2005; 174:2730-2737; doi: 10.4049/jimmunol.174.5.2730
http://www.jimmunol.org/content/174/5/2730
In Vivo and in Absence of a Thymus, the Enforced Expression of the Notch Ligands Delta-1 or Delta-4 Promotes T Cell Development with Specific Unique Effects

Alix de La Coste,* Emmanuelle Six,† Nicolas Fazilleau,‡ Laurent Mascarell,§ Nicolas Legrand,²* Marie-Pierre Mailhé,* Ana Cumano,† Yacine Laâbi,³,4,7 and Antonio A. Freitas⁴,5,*

The role of Notch signaling in T cell commitment during lymphoid development is well established. However, the identity of the ligand that triggers this critical signal in vivo is still unclear. By overexpressing Delta-1 and Delta-4 ligands in the hemopoietic cells of athymic nu/nu host mice, we demonstrate that, in vivo and in the absence of a thymus, Delta-1 or Delta-4 expression is sufficient to promote T cell development from the most immature progenitor stages to complete maturation of both CD8⁺ and CD4⁺ αβ T cells. The mature T cells developing in a Delta-1- or Delta-4-enriched environment express a diverse TCR repertoire, are able to proliferate upon in vitro TCR stimulation, but show different profiles of cytokine production after in vitro anti-CD3 stimulation. The Journal of Immunology, 2005, 174: 2730–2737.

Notch is a highly conserved signaling pathway involved in cell fate choice during development in organisms ranging from invertebrates to mammals. This cell fate determination is mediated by interactions between the Notch receptors and the Notch ligands Delta and Jagged (1). Notch signaling plays a major role in several steps of lymphoid cell commitment and lineage decision (2–5). Induced inactivation of Notch-1 blocks T cell development and allows ectopic accumulation of mature B cells in the thymus (3). Overexpression of an active form of Notch-1 (NotchIC) induced the development of immature double-positive (DP)⁶ CD4⁺CD8⁻ T cells in the bone marrow (BM) with a concomitant inhibition of B cell differentiation (4). Notch signaling has also been shown to favor the αβ vs γδ T cell lineage choice (2, 6).

Four Notch receptors (Notch-1 to -4) and five ligands (Delta-1, -3, and -4, and Jagged-1 and -2) have been identified in mammals. They are differentially expressed during lymphoid development, and their expression is tightly regulated (7). Although there is direct evidence for a major role of Notch signaling in regulating hemopoietic stem cell commitment to the T cell lineage (reviewed in Ref. 5), little is known about the specificity of the ligands that trigger Notch signals in vivo. It has been suggested that distinct downstream events can be activated by specific interactions between different ligands and receptors (8). The dynamic pattern of expression, the potential combinations between ligands and receptors, and the compartmentalization of gene expression, all suggest that a specific signal could induce different effects on development, differentiation, and/or survival (9). Because Delta-1-expressing stromal cell lines can induce CD8 but not CD4 T cell commitment (10) from mouse hemopoietic progenitors, in vitro, Delta-1 might be the critical ligand for CD8 T cell development. Moreover, in vitro stromal cells expressing human Delta-1 but not human Jagged-1 support the development of the T/NK common lymphoid progenitors using human cells (11). However, it was recently shown that the conditional Delta-1-null mice exhibit normal T cell development, suggesting that there is a redundancy of Notch-Delta ligand signaling driving T cell development (12). The best candidate for such redundant or compensatory function is Delta-4, another member of the Delta ligand family, which shares substantial homology with Delta-1. Delta-4, like Delta-1, is expressed in the thymus, and Delta-4-expressing OP-9 stromal cell lines can support development of Thyl⁻ cells in vitro, i.e., induce the commitment of hemopoietic progenitors into T cell lineage (12). Delta-4 overexpression in vivo also leads to the ectopic development of immature CD4⁺CD8⁻ but not of mature T cells in the BM (13, 14). However, whether Delta-1 is capable of driving full T cell development in vivo and whether Delta-1 and Delta-4 effects on T cell development are similar and/or complementary remains unknown.

To better understand the role of Delta ligands during lymphopoiesis in vivo, we reconstituted the immune system of lethally irradiated athymic nu/nu mice with hemopoietic progenitor cells.
retrovirally transduced to overexpress Delta ligands. Our results demonstrate that, in the absence of a thymus, Notch activation by Delta-1 or Delta-4 in vivo induces ectopic T cell development in the BM, spleen, and lymph nodes (LN)s and generates CD8\(^+\) and CD4\(^+\) \(\alpha\)\(\beta\) mature T cells. Moreover, we show that the T cells developed in absence of a thymus are functional, and also that the role of Delta-1 and Delta-4 in T cell maturation is nonredundant because each ligand induces unique specific effects.

**Materials and Methods**

**Mice and fetal liver chimeras**

C57BL/6 (B6) Ly\(^5\) and Ly\(^5\)\(^b\) mice were obtained from the Centre de Développement des Techniques Avancées-Centre National de La Recherche Scientifique (Orl\'ans, France). C57BL/6Ly\(^5\)\(^b\) nu/nu mice were obtained from Janvier (Le Genest St. Isle, France). Six- to 8-week-old B6.Ly\(^5\)\(^b\) host mice were lethally irradiated (900 rad) with a \(153\)Cs source and injected i.v. with 0.3 to 1 \(\times\) 10\(^6\) infected fetal liver (FL) cells. FL cells were from Ly\(^5\)\(^b\) mice. Seven to 9 wk after, mice were sacrificed and BM, thymus, spleen, and LN's were harvested for FACS analysis.

**Delta-1 and Delta-4 constructs**

Full-length murine Delta-like-1 cDNA (Delta-1) (15) was subcloned into the BglII-XhoI sites of the MSCV-IRE-S- GFP vector (MIG) (16), and full-length murine Delta-like-4 cDNA (Delta-4) was subcloned into the XhoI-EcoRI sites of the same vector.

**Cell culture**

Plat-E packaging cells were cultured in DMEM (In vitrogen Life Technologies) supplemented with FBS, penicillin/streptomycin (In vitrogen Life Technologies), sodium pyruvate (In vitrogen Life Technologies), 2-ME (In vitrogen Life Technologies), puromycin, and blasticidin (Sigma-Aldrich) (17). FL cells were cultured in DMEM medium supplemented with FBS, penicillin/streptomycin, sodium pyruvate, 2-ME, and supernatant from IL-7-, c-kit ligand-, and flt3 ligand-producing cell lines (kindly provided by F. Melchers (Institute of Immunology, Berlin, Germany) and R. Rottapel (Department of Immunology, University of Toronto, Toronto, Canada), respectively.

**Retrovirus production and injection of FL cells**

Empty (MIG) or recombinant (Delta-1 and Delta-4) viruses were obtained after transfection of the Plat-E ecotropic packaging cell line (17) using Fugene (Roche Molecular Biochemicals). Retrovirus-containing supernatants were collected 48 h after transfection and used for spin infection of FL cells. FL cells were harvested from embryonic day 14.5 embryos and cultured in 24-well plates for retroviral infection after RBC lysis. In the retroviral system used here, the expression of the inserted cDNA is directly correlated with expression of GFP expression (16). One day after plating, the cells were infected daily for 3 days in presence of IL-7, c-kit, and flt3 (see **Cell culture**). Infection efficiency was \(\sim 60–70\%\) (GFP marker). Delta expression was confirmed by Western blot and RT-PCR analysis (not shown).

**Flow cytometry and sorting**

Single-cell suspensions were prepared in RPMI 1640 medium supplemented with FBS. Cell surface four- and five-color staining was performed using the appropriate combinations of PE, biotin, and allophycocyanin-conjugated Abs (BD Pharmingen), PerCP-streptavidin and PECy7-streptavidin (BD Pharmingen) were used to reveal biotinylated Abs. The following Abs were used: anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-TCR \(\beta\)-chain (H57-597), anti-CD3 chain (145-2C11), anti-CD19 (1D3), anti-CD43 (ST), anti-CD44 (IM7), anti-Thy1.2 (30-H12), anti-IL-2R\(\alpha\)-chain (7D4), anti-B220 (RA3-6B2), anti-Ly\(^5\) (A20), anti-CD45RB (16A), and anti-IgM (II-41). Acquisitions and data analysis were performed with a FACS Calibur or a BD-LSR (BD Biosciences) interfaced to CellQuest software. Analysis was gated on viable cells on the basis of forward and side scatter.

**Immunoscore analysis**

Total RNA was extracted from CD4\(^+\) and CD8\(^+\) sorted splenocytes from Delta-1 and Delta-4 nu/nu reconstituted mice using RNaseasy mini kit (Qiagen) and reverse-transcribed into cDNA using oligo(dT) and SuperScript II (Invitrogen Life Technologies). PCR's were conducted using Taq polymerase (Promega) in the supplier’s buffer. cDNA was amplified using V\(\beta\)-specific sense primers and an antisense primer hybridizing in C\(\beta\) segments. Amplified products were used as template for an elongation reaction with fluorescent tagged oligonucleotides (run-off reactions) as described (18).

**In vitro proliferation assay**

Total or CD4\(^–\) and CD8-depleted spleen cells from MIG, Delta-1, and Delta-4 nu/nu reconstituted mice were incubated in 96-well plates (2 \(\times\) 10\(^4\) cells/well) at 37°C and 5% CO\(_2\) in a final volume of 200 \(\mu\)l in RPMI 1640 supplemented with FBS (ICN), penicillin/streptomycin (Invitrogen Life Technologies), sodium pyruvate (Invitrogen Life Technologies), and 2-ME (Invitrogen Life Technologies). Cells were stimulated by coated anti-CD3e (10 \(\mu\)g/\(\mu\)l; 145-2C11; BD Pharmingen) and soluble anti-CD28 (2.5 \(\mu\)g/\(\mu\)l; 37-51; BD Pharmingen). Cultures were in triplicate. After 2–4 days of culture, cells were pulsed overnight with 1 \(\mu\)Ci per well of \([\text{H}]\) thymidine (ICN Pharmaceuticals) and counted (TOMTec). For CD4 and CD8 proliferation, spleen cells were depleted of either CD8\(^+\) or CD4\(^+\) cells, respectively, using Macs columns (Miltenyi Biotec), and checked for purity before use.

**Cytokine production assay**

Supernatants from anti-CD3e (145-2C11; BD Pharmingen)- and anti-CD28 (37-51; BD Pharmingen)-stimulated CD4 and CD8 T cells were removed after 72 h of culture, and IFN-\(\gamma\), IL-2, IL-4, and IL-5 production assessed by standard ELISA. Ninety-six-well plates were coated using unconjugated anti-IFN-\(\gamma\) (R4-6A2; BD Pharmingen), anti-IL-2 (JES6-1A12; BD Pharmingen), anti-IL-4 (BVD4-1D11; BD Pharmingen), and anti-IL-5 (TRFK5; BD Pharmingen) capture Abs, and detection was performed using corresponding biotinylated Abs (XM1G1.2, JES6-5H4, BV26-24G2, and TRFK4 clones from BD Pharmingen). Biotin was revealed using streptavidin HRP and O-phenylenediamine as substrate. All dosages were performed in duplicate. Results were standardized with recombinant murine cytokines (BD Pharmingen). Cytokine concentrations are expressed in nanograms per milliliter.

**Results**

**Inhibition of B cell development at the pro-B to pre-B transition stage**

To investigate in vivo the specific effects of Delta Notch ligand overexpression on the development of the immune system, we reconstituted lethally irradiated Ly\(^5\)\(^b\) host mice with FL cells from Ly\(^5\)\(^b\) donors transduced with a MIG retrovirus containing the mouse Delta cDNA (Delta-1 or Delta-4), or with the control retrovirus (MIG). FL cell infection efficiency was \(\sim 50–70\%\) (GFP marker), and both GFP\(^+\) and GFP\(^–\) FL cells were injected. Seven weeks after the cell transfer, we studied B and T cell reconstitution in the resulting chimeras.

We found that the B6 host mice reconstituted with Delta-1 FL cells had significantly fewer nucleated cells in the BM, spleen, and LN than control mice (Table I). This was in large part due to a marked decrease in the number of B220\(^+\) CD19\(^+\) B cells (Table I). Although reduced cell numbers were observed in both the immature (B220\(^+\)IgM\(^–\)) and mature (B220\(^+\)IgM\(^+\)) B cell compartments of the BM, the cell loss was more pronounced for the pre-B (B220\(^+\)IgM\(^–\)CD43\(^+\)) and mature B cells than for the pro-B cells (B220\(^+\)IgM\(^+\) CD43\(^+\)) (9-fold vs 2.5-fold, Table I). Furthermore, in the Delta-1 mice, the relative fraction of pro-B cells in the immature B cell pool was 3-fold higher than in control mice (Fig. 1). The pro-B cell population was checked for CD19 expression (data not shown) to exclude the B220\(^+\)CD43\(^+\)CD19\(^–\) population of NK precursors residing in the BM (19). These results indicate that the in vivo enforced expression of Delta-1 leads to a block of B cell differentiation at the pro-B cell stage. As a consequence from the spleen and the LN of Delta-1-reconstituted mice, we recovered a lower number of B cells (31 \(\times\) 10\(^6\) and 1.2 \(\times\) 10\(^6\) for spleen and LNs, respectively) than from control mice (103 \(\times\) 10\(^6\) and 7.7 \(\times\) 10\(^6\), respectively) (Table I). However, the difference of peripheral B cell numbers was less pronounced than in the BM (Table I), suggesting that some cells escape the Delta-1-induced inhibition of B cell development. Indeed, \(~40\%\) of the total host BM cells were
GFP. Partial recovery of B cell numbers in the periphery may also result from homeostatic mechanisms that compensate for a reduced production of B cell precursors in the BM (20).

**Extrathymic localization of immature CD4^+CD8^- DP T cells**

In the BM of the mice reconstituted with Delta-1-overexpressing cells, we observed a population of CD4^+CD8^- DP T cells that is absent in control mice (Fig. 2A). These cells represented 20–45% of the BM nucleated cells (Fig. 2A), and most (80%) of them were CD3eintlow (A, histogram). This ectopically localized population of CD4^+CD8^- DP T cells was also present in the spleen and LN of the Delta-1-reconstituted mice (data not shown), but not in control mice. These cells were donor derived, because they expressed the Ly5.1^+ donor marker (data not shown) and ~20% were GFP^+.

We assessed for the earliest immature stages of T cell development in the BM of Delta-1-reconstituted mice by analyzing the expression of CD44 and CD25 among the Thy1.2^+ T cell precursors after exclusion of B cells (B220^+), DP and mature single-positive (SP) T cells (CD4^- and CD8^- cells) (Fig. 2B). When compared with T cell development in a normal thymus or in the thymus of the same mouse (Fig. 2B, lower panels), we observed that the BM Thy1.2^- cells from the Delta-1-reconstituted mice recapitulated the normal stages of double-negative (DN)—DN1 to DN4—T cell maturation (B, upper panels). This finding indicates that Delta-1 can induce T cell lineage commitment from lymphoid progenitors in the BM.

In the Delta-1 mice, the thymus showed a normal distribution of DN, DP, and SP CD4^- and CD8^- cells. T cell development was observed equally well between both GFP^+ and GFP^- cells (Fig. 2C). Furthermore, we did not observe any significant differences in the other hemopoietic subsets, including myeloid cells, NK cells, and γδ T cells. The absence of differences in the γδ T cell population in the Delta-1- and MIG-reconstituted mice indicated that signal(s) induced by Delta-1, as described for enforced Notch-1 activation (2), favor γδ vs γε lineage commitment.

**Delta-1 and Delta-4 promote complete T cell maturation in the absence of a thymus**

In B6 hosts, the presence of a host thymus does not allow us to determine whether Delta-1 signals alone suffice to induce the ectopic development of CD4^-CD8^- T cells and the complete maturation of CD4^+ and CD8^- SP T cells. Indeed, in wild-type mice, the CD4^-CD8^- T cells ectopically localized in the BM and peripheral organs could be thymus-derived cells that migrate into and expand in the peripheral tissues. To exclude this possibility, we reconstituted lethally irradiated nu/nu mice (21) with FL cells infected with Delta-1 or control retrovirus and analyzed the resulting chimera. Athymic nu/nu hosts lack thymic epithelium due to disruption of the Whn gene (22), and thus do not develop populations of thymus-dependent T cells in their secondary lymphoid organs, i.e., spleen and LNs (21). Because Delta-4 overexpression has been shown to induce the ectopic development of immature CD4^-CD8^- DP T cells in the BM (13, 14), we decided to compare the effects of Delta-1 and Delta-4 overexpression on lymphoid development in the absence of a thymus. In the reconstituted Delta-1 and Delta-4 athymic hosts, 25–35 and 10–20% of the total BM cells, respectively, were GFP^+.

In the athymic nu/nu mice overexpressing Delta-1 and Delta-4, B cell development was inhibited at the pro-B to pre-B transition (not shown) as in B6 mice (see above). B cell inhibition was markedly more pronounced in the Delta-4 mice with an almost complete absence of mature B cells in the BM (1.2 × 10^6 for Delta-4 BM, 2.5 × 10^6 for Delta-1, and 8 × 10^6 for MIG-reconstituted mice) and very few B cells in the spleen (5 × 10^6 for Delta-4 spleen, 18 × 10^6 for Delta-1, and 80 × 10^6 for MIG-reconstituted mice).

Development of ectopic CD4^-CD8^- DP T cells occurred in BM, spleen, and LN of the Delta-1- or Delta-4-overexpressing athymic nu/nu mice (Fig. 3, shown for LNs) but not in control mice (Fig. 3). These DP cells were both GFP^+ and GFP^- and all expressed low levels of the TCR β-chain and CD3ε (data not shown). However, there were quantitative differences between the mice overexpressing the two Delta ligands. Thus, whereas in the Delta-1-overexpressing mice, the number of DP T cells was relatively low—<5 × 10^6 in the spleen and LN—in the Delta-4 mice, T cell development was associated with a strong increase in the
number of CD4⁺CD8⁻ DP T cells—80x10⁶ in the spleen and 40x10⁶ cells in the LNs. In the spleen (data not shown) and LN (Fig. 3) of both the Delta-1 and Delta-4-overexpressing nu/nu mice, we identified populations of mature SP CD4⁺ and SP CD8⁺ T cells (Fig. 3, lower panels), comprising GFP⁺ and GFP⁻ cells (not shown), which were absent from control mice (upper panel). The mature SP T cells were TCRβ chain⁺ (data not shown) and CD3ε⁺ (Fig. 3, histograms); in other words, they expressed αβTCR. It should be noted that the CD3ε expression levels were lower in CD8 than in CD4 T cells in both Delta-1 and Delta-4 mice as in wild-type B6 mice. They were all of donor origin, i.e., they expressed Ly5.1⁺ and therefore not related to the host thymus-independent T cells that are produced and present in the intestinal epithelium of nude mice (data not shown). Despite the different number of DP T cells, the number of mature SP mature T cells recovered in the Delta-1 or Delta-4 hosts was identical, and the number of DP T cells, the number of mature SP mature T cells, and the number of peripheral mature T cells recovered was low compared with those obtained in an intact mouse. In both Delta-1 and Delta-4 nu/nu mice, there were ~1 to 6x10⁶ mature peripheral SP CD4⁺ T cells and 1 to 4x10⁶ mature SP CD8⁺ T cells (1-4% of the total number of spleen cells and 2-7% of the total number of LNs cells) (Fig. 3). The low number of mature T cells recovered in the Delta chimeras suggest that the T cell maturation induced by the overexpression of Delta-1 and Delta-4 in absence of a thymus was not as efficient as that induced by the normal thymus environment.

We studied the TCR-Vβ repertoire of the mature T cells recovered in the athymic mice overexpressing Delta. Using the immunoscope method to analyze the sizes of the hypervariable CD3R3-like regions of the β-chains for all possible Vβ-Cβ combinations in purified CD4⁺ and CD8⁺ T cells from Delta-1 (Fig. 4A) and Delta-4 (B) mice (18), we found that their TCR repertoires though using a complete set of Vβ genes (Fig. 4, A and B) are less diverse than that of normal B6 mice, i.e., they are oligoclonal. It should be pointed out that each individual Delta mouse expressed a unique pattern of the Vβ-chain CD3R3-like regions (not shown). We have previously shown that oligoclonal repertoires correlate with low rates of thymus T cell production (23). Peripheral activation and expansion of some T cell clones are homeostatic mechanisms that compensate for the low T cell production in central compartments and that lead to a less diverse repertoire (23). Confirming this hypothesis, in Delta-1 and Delta-4 nu/nu mice, most CD4⁺ T cells are CD45RB⁺ (Fig. 4C) and CD8⁺ T cells are CD44⁺ (D), i.e., mature CD4⁺ and CD8⁺ T cells in the Delta mice express late activation markers.

Functional characterization of T cells developed in absence of thymus: Delta-1 and Delta-4 promote different pattern of cytokine production

To test the functional capacity of the mature CD4⁺ and CD8⁺ T cells, we studied their proliferation and cytokine production upon anti-CD3 and anti-CD28 in vitro stimulation. Upon TCR stimulation, CD4⁺ (Fig. 5A) and CD8⁺ (B) lymphocytes from nu/nu mice overexpressing Delta-1 and Delta-4, proliferate as well as mature T cells from B6 mice (A and B). This finding indicates that the T cells, which develop in absence of a thymus, are functionally mature (Fig. 5). Concerning cytokine production, we found that both CD8⁺ and CD4⁺ T cells from Delta-1 and Delta-4 nu/nu mice produce IFN-γ at levels similar to those of B6 mice (Fig. 6). Surprisingly, the IL-2 production by both CD8 and CD4 T cells developed in the athymic Delta-1 and Delta-4 mice was dramatically lower than that obtained with T cells from wild-type mice (Fig. 6). More importantly, the CD4⁺ T cells from nu/nu mice overexpressing Delta-4 produced high levels of IL-4 and IL-5 (Fig. 6B), whereas CD4⁺ T cells from the nu/nu Delta-1-reconstituted or B6
mice failed to produce detectable levels (80 pg/ml) of these cytokines (B). Overall, these findings suggest that mature T cells developed in an environment overexpressing Delta-1- or Delta-4-induced Notch signaling are not similar.

**Discussion**

In the present study, we found that the in vivo environmental enforced expression of the Notch ligands Delta-1 and Delta-4 during hemopoiesis blocks B cell differentiation and promotes T cell development in the absence of a thymus.

In hemopoietic chimeras overexpressing the Delta ligands, we observed an inhibition of B cell development, due to a block at the pro- to pre-B cell transition stage. It has been reported that the expression of a constitutively active form of Notch-1 in hemopoietic cells inhibits B cell production (4) and that the conditional Notch-1 inactivation leads to ectopic B cell development in the thymus (3). In vitro studies of hemopoietic stem cell differentiation have also shown that Notch-1 activation leads to a complete absence of the B lineage (10 –12). In our Delta chimeras, the presence of some B cells may be due to the fact that, within the BM environment, not all relevant stromal cells express the Delta ligands, allowing some cells to escape and engage in the B cell lineage pathway. Because it has been reported that pro-B cells express only small amounts of Notch-1 (24), the differential effects observed in the number of pro-B and pre-B cells suggest that in vivo Delta-1 may preferentially interfere at the pro-B to pre-B transition possibly by acting on Notch receptors other than Notch-1. Notch-2 is the most highly expressed Notch receptor in B lineage cells (25), and the expression of an activated form of Notch-2 permits early pro-B cell development, but blocks B cell maturation at the pre-B stage (26). Pro-B cells may need to down-regulate Notch-2 receptor expression to continue through B cell development (26). Like Delta-1, Delta-4 overexpression also blocks B cell development at the pro-B stage (not shown). B cell inhibition in presence of Delta-4 in athymic nu/nu mice was more pronounced than in Delta-1 mice (data not shown). Overall, these results in accordance with previous results show that Notch-induced inhibition of B cell differentiation occurs only on committed B cells (12). The partial recovery of B cell numbers observed in the periphery of the Delta chimeras may result from homeostatic mechanisms that compensate for the diminished production of B cells in the BM (20).

In the BM of B6 mice overexpressing Delta-1, we detected the ectopic development of a population of immature DP T cells. Mice overexpressing a constitutively active form of the Notch-1 receptor showed an expansion of immature DP T cells that developed independently of the thymus (4). In these latter studies, however, the use of thymectomized mice (4), which retain sizeable populations of host mature T cells, did not allow determination of whether T...
FIGURE 6. Cytokine production profiles. CD8-enriched spleen cells (CD4\(^+\) depleted) and CD4-enriched spleen cells (CD8\(^+\) depleted) from Delta-1 nude, Delta-4 nude, and wild-type B6 mice were stimulated in vitro with anti-CD3 and anti-CD28. Cytokine production was tested by ELISA in the culture supernatants after 3 days of in vitro culture. IFN-\(\gamma\) and IL-2 production is shown for CD8-enriched spleen cells (A) and IFN-\(\gamma\), IL-2, IL-4, and IL-5 production for CD4-enriched spleen cells (B). Production of IL-2 was identical after 2 days of culture (not shown). The cytokine concentrations are expressed in nanograms per milliliter. Cytokine production by spleen cells from control MIG nude mice is also shown for background level. Similar results were obtained in two distinct experiments.

diverse TCR-V\(\beta\) repertoire, which a priori exclude a neoplastic monoclonal transformation of the mature T cells. The phenotypical differences observed between Delta-1 and Delta-4 chimeras could also be due to quantitative and not qualitative effects. However, we obtained similar T cell phenotypes in mice with different fractions (ranging from 10 to 50\%) of GFP\(^-\) Delta-expressing cells, suggesting that the differences observed are more likely due to qualitative rather than quantitative differences. Finally, we should stress that in the in vitro analysis of T cell development using the stromal cell line OP-9 overexpressing Delta-1 or Delta-4 did not highlight any differential effects between those two ligands (10, 12), strongly reinforcing the need of the in vivo studies performed here to determine the specific unique effects of each Notch ligand.

We show that, in the absence of a thymus, the expression of both Delta-1 and Delta-4 ligands suffice to induce the complete maturation of CD8\(^+\) and CD4\(^+\) \(\alpha\beta\) mature T cells. Because T cells were both GFP\(^-\) and GFP\(^+\), we conclude that the observed effects are due to an environment rich in Notch ligands and not determined by the expression of the ligand on developing T cells themselves. Thus, as to the question whether Notch ligands act on cis or trans, our findings demonstrate that Delta ligands can act on trans, but do not permit the conclusion of a possible cis effect. In all, our findings extend and complete previous in vitro observations reporting that Delta-1 or Delta-4 overexpression on OP-9 stromal cells provide the signals necessary for the maturation of SP CD8\(^+\) cells but not of SP CD4\(^+\) cells (10, 12). In our in vivo experiments, however, both CD4\(^+\) and CD8\(^+\) SP cells developed, a difference that is most likely due to the presence in vivo of MHC class II molecules, which are not expressed by OP-9 cells. The finding that, in Delta-1 and Delta-4 null mice, the CD4/CD8 T cell ratio is the same as in normal mice suggests that Notch activation via Delta ligands is not involved in CD4/CD8 lineage decision. Despite the significant number of DP T cells recovered, which are significantly higher in the presence of Delta-4, the number of mature peripheral T cells was low and identical in both Delta-1 and Delta-4 chimeras, i.e., ~10\% of those of a wild-type mouse. The TCR-\(\beta\) chain repertoires of both CD4\(^+\) and CD8\(^+\) SP T cells were less diverse than those of T cells from a normal mouse, and most mature T cells express an activated phenotype. These observations suggest that T cell maturation induced by the Delta ligands in the absence of a thymus may follow different rules and is less efficient than in the normal thymus environment. In the thymus during T cell differentiation, the Notch modifier Lunatic Fringe (28) modulates Notch signals (29). In the absence of a thymus and of Lunatic Fringe modulation, the constitutive expression of Delta ligands might induce a state of continuous T cell activation. In absence of the specialized thymus medulla epithelial cells, negative selection is defective (30, 31), which may result in the accumulation of self-reactive T cells expressing an activated phenotype. The increased fraction of activated T cells may also result from homeostatic mechanisms that compensate the lower rate of new T cell production (23).

Altogether, these results suggest that members of the Delta Notch ligand family are likely to play a crucial role in T lineage commitment in vivo. Delta-1 and Delta-4 could act synergistically to direct T cell commitment. The observation that T lymphocytes developed normally in Delta-1 conditional knockout mouse (12) suggest that Delta-1 function can be replaced during adult T cell development. Study of the thymus of Delta-4 null mice will be necessary to clearly establish whether Delta-1 and Delta-4 share overlapping functions during T cell development. However, our present findings indicate that Delta-1 and Delta-4 are not redundant because they show different and unique effects on T cell maturation. In the absence of a thymus, mature T cells developed in...
the presence of Delta-1 or Delta-4 show unique patterns of cytokine production after in vitro stimulation. Thus, although all T cell populations studied secreted IFN-γ, Delta-1- or Delta-4-induced T cells produced very low levels of IL-2 in contrast to wild-type B6 cells. The lack of IL-2 production is intriguing and remains to be elucidated. This could be attributed to the state of T cell activation, but naturally activated T cells secrete IL-2 upon in vitro stimulation (C. Tanchot, unpublished observations). More interestingly, the populations of CD4 T cells developed in presence of Delta-4 produce IL-4 and IL-5, whereas CD4 T cells developed in the presence of Delta-1 or in control mice did not. IL-4/IL-5 production is associated with Th2 differentiation, whereas IFN-γ production is usually associated with Th1 functions (32). The T cells from Delta-4 mice do not show such clear-cut polarization because they produce IFN-γ, IL-4, and IL-5 at a population level. In vitro co-stimulation of TCR-activated T cells with a soluble form of Delta-1 increases the number of cells secreting IFN-γ (33), whereas cocultures of naive transgenic CD4+ T cells and APCs expressing Delta-1 or Jagged-1 ligands, suggested that Delta and Jagged ligands promote Th1 and Th2 differentiation, respectively (34). We show in this study that an in vivo Delta-4 promotes IL-4 and IL-5 secretion. These discrepancies may be due to the different experimental systems used. Although in the previous study cytokine production was tested by short-term exposure of naive transgenic CD4+ T cells to APCs expressing different Notch ligands (34), we studied cytokine production by T cells that develop in the absence of thymus in an environment enriched for the Delta-1 and Delta-4 Notch ligands. It remains to be established whether Delta ligands induce an irreversible T cell polarization, whether the cytokine changes induced are intrinsic to the T cells or determined at the periphery by the D1- and D4-expressing APCs, or whether GFP+ and GFP− T cells show similar cytokine patterns. From our results on the differential effects of Delta-1 and Delta-4 on T cell maturation, it is tempting to speculate that Delta-1 and Delta-4 could act on different Notch receptors. Delta-4 may preferentially signal on different Notch receptors. Delta-4 may preferentially signal a murine gene closely related to Notch2 potentiates CD8 lineage maturation and promotes the selective development of B1 B cells.

Acknowledgments
We thank A. Almeida, R. S. Wildin, A. Israel, A. Strasser, and F. Logeat for helpful comments and discussion. We thank T. Kitamura for providing the Plat-E cell line, A. Gossler for the mouse Delta-1 cDNA, and D. Henrique for the mouse Delta-4 cDNA.

Disclosures
The authors have no financial conflict of interest.

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